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DNA methyltransferase 3A characterization in clonal hematopoiesis, aging and acute myeloid leukemia

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DNA METHYLTRANSFERASE 3A CHARACTERIZATION IN CLONAL HEMATOPOIESIS, AGING AND ACUTE MYELOID LEUKEMIA

by

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ABSTRACT

The expected growth of the older adult population in the United States over the next several decades will have an unprecedented impact on the health care system. Research has shown that age is the greatest risk factor for developing cancer and certain hematological malignances. Insights on acute myeloid leukemia have suggested that premalignant somatic mutations in stem cells are responsible for age associated medical conditions. Persistence of preleukemic clones and the risk of relapse is linked with an abnormal DNA methyltransferase 3A (DNMT3A) gene especially in the R882H region which may lead to a phenomenon known as clonal hematopoiesis. The DNMT3A gene provides instructions for making an enzyme that establishes DNA methylation patterns and is believed to form the initial mutation in acute myeloid leukemia. Because of the significance of DNMT3A mutations in the pathogenesis of leukemias and clonal hematopoiesis with respect to the geriatric population, the goal of the thesis was to generate DNMT3A proteins for future research via a bacterial expression vector. Wildtype and dominant negative DNMT3A proteins were not successfully generated, but the study is still an ongoing process. The overview of the entire long term study is to focus on the mechanism insight on DNMT3A activity and its contribution to acute myeloid leukemia development.

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LIST OF ABBREVIATIONS

ALL.....Acute Lymphocytic Leukemia

AML..... Acute Myeloid Leukemia

bp..... Base Pair

CH.....Clonal Hematopoiesis

CHIP ..... Clonal Hematopoiesis of Indeterminate Potential

ChIP .....Chromatin Immunoprecipitation

CLL..... Chronic Lymphocytic Leukemia

CML..... Chronic Myeloid Leukemia

DNMT3A.....DNA (Cytosine-5)-Methyltransferase 3A

dNTP.....Deoxyribonucleotide Triphosphate

HSC.....Hematopoietic Stem Cells

LB .....Lysogeny Broth

NCI..... National Cancer Institute

PCR..... Polymerase Chain Reaction

PWWP.....Pro-Trp-Trp-Pro

SEER.....Surveillance, Epidemiology, and End Results

TET2 ..... Tet Methylcytosine Dioxygenase 2

VAF.....Variant Allele Frequency

WHO.....World Health Organization

WT……....Wild Type

CHAPTER 1

INTRODUCTION

1.1 Overview

Leukemia is a complex disease, but a path forward to potentially cure it is revealed with a surprising connection between hematological malignancies and the clonal hematopoietic process associated with age (Steensma 2018). Alterations in cellular genes - which accumulate over time - are the main drivers in cancer (Risques & Kennedy, 2018). The acquisition of multiple mutations in oncogenes and tumor suppressor genes alter cellular division. Mutations in epigenetic modifying genes such as DNMT3A and TET2 are age related and partially responsible for clonal hematopoiesis of indeterminate potential which is link to acute myeloid leukemia (AML) (Buscarlet *et al*., 2017). Since the geriatric population has accumulated more DNA errors over a period of time, the likelihood of a benign clonal hematopoietic stem cell turning cancerous increases exponentially, specifically in hematological systems (López-Otín *et al*., 2013).

There is a convergence of concepts related to acute myeloid leukemia. Not only is it an age related disease, but AML is a notable exception to most cancers as it possesses relatively few mutations across the genome and has unique epigenetic signatures (DiNardo & Cortes, 2016). Because of certain defining features of AML, the nature of the disease forms the basis of cancer research and clonal hematopoiesis. A thorough comprehension of AML requires familiarity of blood formation and leukemia classification with an emphasis to the geriatric population. In addition, knowledge of clonal hematopoiesis of indeterminate potential along with a brief background in cancer

and epigenetics is helpful to better characterize the complexities of AML. Because the thesis is rather large in scope, the introduction is organized into sections.

1.2 Clonal Hematopoiesis

Clonal hematopoiesis of indeterminate potential (CHIP) is a syndrome primarily observed in the elderly (Park & Bejar, 2018). It is an age-related phenomenon in which hematopoietic progenitors contribute to the creation of a genetically diverse subpopulation of blood cells (Jaiswal *et al*., 2014). Because of the evolving nature associated with clonal hematopoiesis, it is linked to an increased risk of cancer and cardiovascular disease (Argüelles *et al*., 2019). Those with CHIP have commonly detectable variants in epigenetic modifying genes such as DNMT3A and TET2 (Buscarlet *et al*., 2017). However, CHIP is fairly benign (Park & Bejar, 2018). The clinical implications of CHIP are not well understood as it has a low association to malignant diseases (Jaiswal *et al*., 2014). Although it is straightforward to screen those for CHIP, therapeutic interventions are absent. Previous research about CHIP suggest it is a proinflammatory marker and a potential diagnostic indicator to certain illnesses (Jaiswal & Libby, 2019).

Normal hematopoiesis in adults is the result of a collective contribution of blood cell production to maintain steady state levels in the peripheral circulation. In a healthy individual, approximately 10^{11}-10^{12} new blood cells are formed daily from hematopoietic stem cells (HSC) (Bouhassira & Vyas, 2015). These HSC usually contribute equally to blood cell production, yet in an abnormal state one HSC gives rise to an outsized proportion of blood cells. For instance, a single cell may birth > 10 percent of blood cell

production (Bouhassira & Vyas, 2015). The expansion of one lineage of cells, or a clone, at a scale disproportionately greater than other clones is known as clonal hematopoiesis (CH) (Bowman *et al*., 2018). It has a low risk factor for developing a hematologic malignancy (see Figure 1) (Sano *et al*., 2018). However, CH is characteristic of leukemias and the prevalence of both increase with age (Bowman *et al*., 2018). The belief is that over the human life span, somatic DNA mutations accumulate in healthy tissues (Steensma 2018). Although most mutations are inconsequential, some give a relative fitness advantage on a single stem cell and its offspring (Steensma & Ebert, 2019).

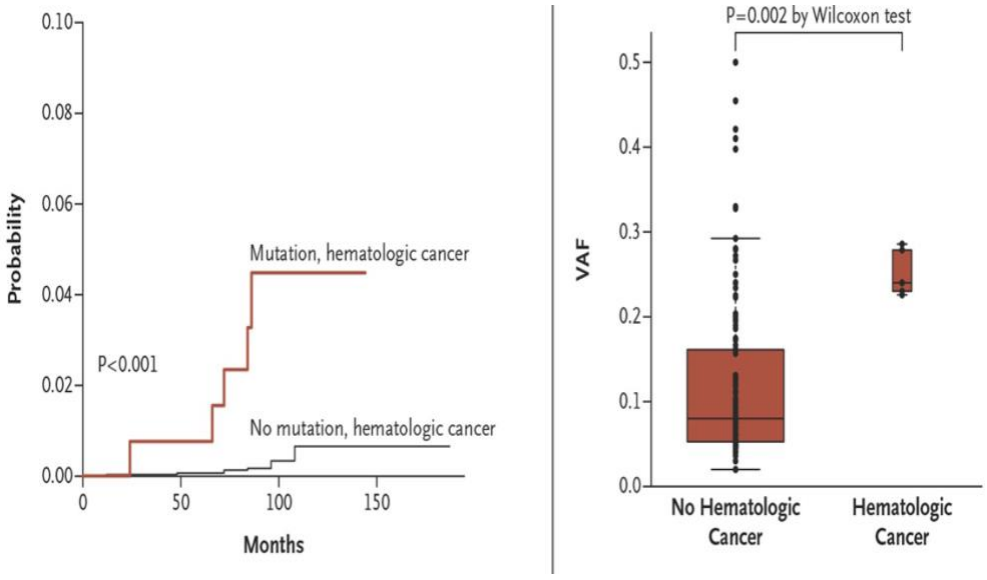

Figure 1: Clonal Hematopoiesis – A Pathway to Cancer. Clonal hematopoiesis increases the risk of hematological malignancy, but the probability is low. Individuals with clonal hematopoiesis have a 5% chance of developing leukemia. (Adapted from Jaiswal *et al*., 2014)

Clonal hematopoiesis has led to a whole new field of medicine for the elderly. Large population studies have identified that CH gradually increases with age (Park & Bejar, 2018). Detectable somatic mutations were rare in individuals younger than 40

years of age but became more frequent with each decade of life thereafter (Risques & Kennedy, 2018). Among individuals greater than 70 years of age, it is estimated that 9.5% of them have variants in genes linked to clonal hematopoiesis (Jaiswal *et al*., 2014). Furthermore, individuals with CHIP tend to progress to adverse outcomes at a rate of about 0.5 to 1% per year (Jaiswal *et al*., 2014). Retrospective and prospective cohort studies of 60 – 70 year old adults suggest that CH is relevant for cardiac disease in the elderly with a 4 – fold higher risk of myocardial infarction (Fuster & Walsh, 2018). Nonetheless, the amount of clonal hematopoiesis (VAF %) required to increase the risk of age-related diseases is not known. Moreover, who is at risk and how does CH evolve?

CANCER

1.31 Cancer Overview

Major advances have been made unraveling the etiologies of numerous cancers. Yet the precise cause(s) of cancer remain elusive (Blackadar 2016). Percivall Pott was the first scientist to demonstrate that cancer may be caused by an environmental carcinogen (Benmoussa *et al*., 2019). However, American Nobel Prize winning Francis Peyton Rous suggested in the 1960s that cancer was the result of a virial transmission (Kumar & Murphy, 2013). These theories are accurate, but incomplete. Ten years later, the term proto-oncogene was back in the scientific spotlight (Kathuria *et al*., 2010). It was believed that certain stressors on DNA active endogenous genes have the potential to cause cancer (Macheret & Halazonetis, 2015). Although these theories help characterize the nature of cancer, they are helpless in explaining even the simplest inquiries of the

disease. Who gets cancer and why? How come it is challenging to treat despite chemotherapeutic medications? What is the precise etiology of cancer? Recent CHIP research may prove useful in elucidating these frequent and important questions, especially in the elderly.

The frontier of cancer research is evolving towards analyzing the precursor conditions leading to the disease (Wacholder 2013). The cure for cancer - as indicated by Siddhartha Mukherjee in his Pulitzer Prize winning book *The Emperor of All Maladies: A Biography of Cancer* - begins with its prevention. How can we cure a disease with a clonal evolving nature? It commences with analyzing the cells and environment that precipitate the trigger of cancer (Wacholder 2013). However, the knowledge to retrace and map these events leading to pathology are murky. Current studies suggest that the architecture of cancer begins with an early indolent pathological state such as CHIP (Steensma 2018). Clonal hematopoiesis may help identify molecular cancer drivers that contribute a growth advantage to certain stem cells. Nonetheless, individuals rarely experience symptoms of CHIP because these mutations are found within normal hematopoietic cells (Genovese *et al.*, 2014). Nevertheless, as one ages, he or she develops and acquires more mutations (see Figure 2), thus illuminating the age related component of cancer (Milholland *et al.*, 2015).

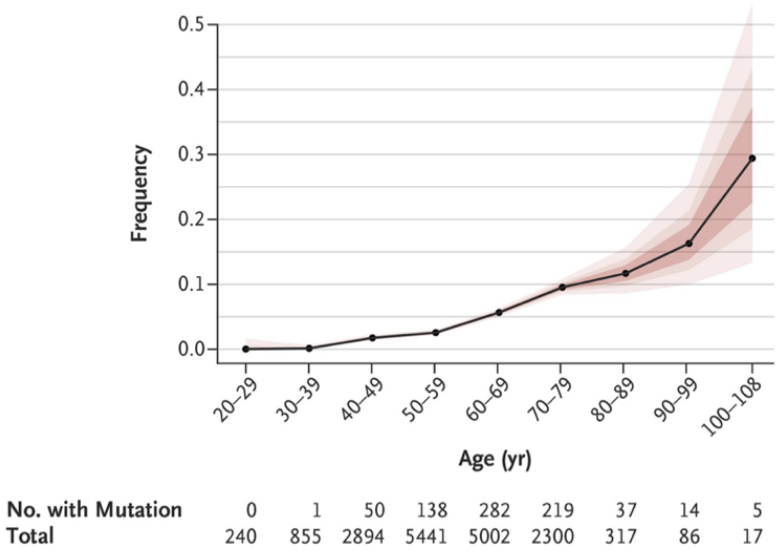

Figure 2: **Prevalence of Somatic Mutations as a Function of Age**. The frequency of mutations increases with age in the general population. (Adapted from Jaiswal *et al*., 2014)

1.32 Genetics of Cancer

Cancer is a multistep disease as single mutations are not sufficient to produce illness (Jackson & Loeb, 1998). The average cancer cell genome has hundreds and sometimes thousands of mutations (Anandakrishnan *et al*., 2019). Amongst all those mutations found, approximately 5 – 20 are in oncogenes and tumor suppressor genes (Martincorena 2017). Hence, it is likely that only a few mutations (driver genes) are participating in the development of cancer. The remainder are simply known as passenger mutations or silent mutations as they occur during the lifetime of the disease (Stratton *et al*., 2009). Hence, these mutations are carried with the driver genes and are not contributing to the cancer phenotype. The analysis of the cancer genome is very complicated as it difficult to distinguish between a passenger or driver mutation (Pon &

Marra, 2015). Fortunately, because of modern technological advancement, scientists are better able to locate and analyze important cancer genes (Stratton *et al.*, 2009).

An oncogene is a gene that when mutated has the potential to cause cancer (Stratton *et al.*, 2009). These genes are responsible for cell growth and proliferation – the essence of a tumor. Most normal cells will undergo a controlled program cell death known as apoptosis (Shortt & Johnstone, 2012). There are a few mechanisms that switch normal cells into cancer including but not limited to replication errors and chromosomal segregation defects. Mutations seen in oncogenes are typically dominant and gain of function (Stratton *et al*., 2009). Furthermore, they increase proliferation and decrease cell death. The first of these oncogenes was observed by Francis Peyton Rous in oncogenic viruses such as HPV (Kumar & Murphy, 2013). When uncorrupted, oncogenes are vital to normal cell division processes and thus are evolutionarily advantageous (Shortt & Johnstone, 2012; DeGregori 2014). However, in the context of cancer these genes become altered (Stratton *et al*., 2009).

Tumor suppressor genes – "antioncogenes" – are important for cancer regulation. They are responsible for slowing down cell division and repairing errors in DNA transcription (Stratton *et al*., 2009). These genes as such are inactivated during cancer in contrast to oncogenes. In addition, tumor suppressor genes are recessive (Payne & Kemp, 2005). Hence, a mutation is needed on both pairs of homogenous chromosomes to cause cancer (Knudson hypothesis) or via a chromosomal non-disjunction with the mutant copy (Paige 2003). Most tumor suppressor genes are acquired spontaneously and are not inherited (Stratton *et al*., 2009). However, those with familial cancer syndromes have a

predisposition to certain cancers due to a family history of inherited abnormalities of tumor suppressor genes. For example, those with a TP53 gene mutation are acquired and represent deficits in half of all human cancers (Rivlin *et al*., 2011).

1.33 Conventional and New Therapeutic Approaches

The most effective and oldest form of cancer therapy is surgery (DeVita & Chu, 2008). If the tumor and the nearby tissue are removed before metastasis, the patient is generally cured (Tohme *et al*., 2017). Depending on the location and grade of the tumor, radiation is a popular choice of treatment. Ionizing radiation beams focus on cancer cells thus damaging them in the process (DeVita & Chu, 2008). The next most common technique to treat cancer is chemotherapy (DeVita & Chu, 2008). When the cancer has spread, radiation is ineffective because the cancer cells are not localized. Chemotherapy drugs are able to diffuse throughout the body systems and can eliminate the cancer cells (Dewhirst & Secomb, 2017). Radiation and chemotherapy both function by inducing DNA damage or inhibiting mitosis (Arruebo *et al*., 2011). While these agents are effective in causing tumor death, some normal cells are sensitive to radiation and chemotherapy (Baskar *et al*., 2012). Hence, unwanted side effects to treatment are extremely common in cancer patients. In addition, many cancers still persist despite medical treatment. Although radiation and chemotherapy may shrink the tumor, some cancer cells remain resistant and potentially metastasize (DeVita & Chu, 2008).

There are several new approaches to cancer therapy. These are anticancer agents targeted and specific to a certain subtype of the disease (Pucci *et al*., 2019). Some targeted therapies block the action of certain enzymes that carries a mutation involved in

tumor growth (Arruebo *et al*., 2011). Another form of targeted therapy includes immunotherapy, particularly monoclonal antibodies. Small-molecule drugs such as angiogenesis inhibitors keep tissue around the tumor from forming blood vessels (Pucci *et al*., 2019). Examples of targeted therapy include breast, colorectal, and lung cancer (Tsimberidou 2015). Although targeted therapy is preferred, it is often not a viable treatment. Researchers are studying many new drugs while personalized medicine specifically for cancer treatments is imminent (Krzyszczyk *et al*., 2018).

GERIATRIC MEDICINE

1.41 Overview

Geriatric medicine is one of the fastest growing specialties in healthcare (Brummel & Ferrante, 2017). As the worldwide population ages, the healthcare community and economy will face significant challenges to meet the needs of the elderly (Knickman & Snell, 2002). According to the United States Census Bureau website, the number of Americans > 65 years of age is expected to double from 53 million currently to 95 million in 2060 (Vincent & Velkoff, 2010). In addition, *An Aging World*, a Census Bureau report co-authored by Wan He, expects that by 2050 those aged 65 and over will significantly outnumber children driven by the large baby boom generation. Because aging is associated with decrease health, chronic conditions such as heart disease, stroke, and cancer will become exponentially more prevalent in the next several decades (Brunilda *et al*., 2020). Simultaneously, healthcare professionals will need to address comorbidities instead of single diseases (Karlamangla *et al*., 2007). Managing these

conditions will increase the financial demands of the healthcare system (Cortaredona & Ventelou, 2017).

The health challenges faced by the elderly are more difficult than younger adults. The elderly are subject to polypharmacy and multiple medical disorders (Maher *et al.*, 2013; Karlamangla *et al*., 2007). The Centers for Disease Control (CDC) and the National Counsel of Aging at the NIH approximate that of those 65 years of age or older, 80% of them have at least one chronic disease and 77% have at least two ailments (National Counsel of Aging Factsheet). In addition, 60% of all cancers and 70% of cancer related deaths occur with the elderly (Cinar & Tas, 2015). The International Cancer Research Institute estimates an increase of 22 million cases of cancer within the next two decades. Currently, these chronic diseases account for three quarters of the United States healthcare (Barrett *et al*., 2016). There is increasing data to suggest that toxicity to cancer treatment is more severe to the elderly (Repetto 2003). Frailty syndrome, which carries an increased risk for poor outcomes, is considered a culprit of these current statistics (Xue 2012). A decline in health and function is seen in older adults (Brunilda *et al*., 2020).

Although aging is a risk factor for developing cancer, it is a natural part of life (Brunilda *et al*., 2020). Because of the improvements in health and medicine, the challenges of an aging population are becoming an issue (Christensen *et al*., 2009). As one ages, there is increasing genomic instability, telomere attrition, and epigenetic alterations (López-Otín *et al*., 2013). DNA replication errors continue to accumulate in the aging process as these mistakes are passed down from each generation of cells

(Milholland *et al.*, 2015). In addition, telomeres – which play a central role in cell fate – become dysfunctional leading to cancer (Gunes *et al.*, 2018). Inadequate telomere repair is associated also with a variety of diseases including several hematological malignancies like acute myeloid leukemia (Kishtagari & Watts, 2017). Telomere maintenance has an important function for pluripotent and multipotent stem cells with regards to clonal hematopoiesis. The expansion of these cells is halted with telomere shortening and altered telomere regulatory mechanisms (Brümendorf & Balabanov, 2006).

EPIGENETICS

1.51 Overview

Interest in the field of epigenetics has increased rapidly with the term becoming more identifiable in the scientific literature and even the main stream media. Different descriptions have been ascribed to the term over the years, thus blurring the definition. British embryologist Conrad Waddington coined the term *epigenetics* in the 1940s to categorize the integrated knowledge of genes and genetics (Deans & Maggert, 2015). His definition was used to denote the poorly understood mechanisms by which a fertilized ovum develops into a mature, complex organism. Epigenetics to Waddington was not very different from embryology and more importantly had no molecular insights to consider (Deans & Maggert, 2015). However, with the benefits of modern insight and increased knowledge of gene expression, the definition of epigenetics has been refined and narrowed.

The term epi is derived from Greek prefix meaning upon or above. Up-to-date knowledge of epigenetics refers to the "post-genomic" era. In spite of the completion of the Human Genome Project – which successfully sequenced every gene in the human organism – gene expression control is not adequately explained without an epigenetic perspective. Every cell in the organism contains the same genetic code, yet cells in different tissues have unique structure and functions (Rasmussen 2003). Epigenetics seeks to understand how this happens. It focuses on the surrounding structure of DNA that control the expression and heritability of genes (Deans & Maggert, 2015). Chemical tags such as covalent modifications allow certain genes to be turned on or off (Kanherkar *et al*., 2014). These epigenetic modifications may bring lasting changes to gene expression without altering the underlying DNA sequence and therefore the genetic code (Handy *et al*., 2011).

1.52 Molecular Basis of Epigenetics

The molecular basis of epigenetics is a complex phenomenon that involves the activation or repression of certain genes without altering the underlying genetic sequence of DNA. (Plass *et al*., 2008). The machinery involved in these modifications enables differentiated cells to have their own separate structure and function (Rasmussen 2003). Most epigenetic changes occur only during mitosis, while some are transmitted to the offspring of the organism through a process called transgenerational epigenetic inheritance (Heard & Martiensenn, 2014). Specific epigenetic processes include bookmarking, imprinting, paramutation, gene silencing, and chromosome X-inactivation (Moosavi & Ardekani, 2016; Handy *et al*., 2011). These phenomena often define and

dictate the faith of the cell as well as the organism. A wide range of molecular biological techniques such as chromatin immunoprecipitation and bisulfite sequencing are employed to decipher these mechanisms. The molecular targets of epigenetic processes include DNA, histones, miRNAs, sRNAs, and prions (Choudhuri *et al*., 2010).

1.53 Epigenetic Regulators in AML – DNMT3A and TET2

DNA (cytosine-5)-methyltransferase 3A (DNMT3A) is an enzyme that is involved in *de novo* DNA methylation. It is part of a family of DNA methyltransferase enzymes that include DNMT1 and DNM3B (Yang *et al*., 2015). The protein structure of DNMT3A comprises of three highly conserved domains: the Pro-Trp-Trp-Pro (PWWP) domain, the ATRX-DNMT3-DNMT3L (ADD) domain, and the catalytic methyltransferase domain (Yang *et al*., 2015). Animal studies in mice have revealed the biological significance of DNA methylation as it is shown that targeted deletion of all DNMT enzymes results in lethality (Plass *et al*., 2008). Moreover, DNMT3A-/- mice have an increase in self-renewal cell division (clonal hematopoiesis) – a hallmark of cancer (Mayle *et al*., 2015). Although these mutations are seen in cancer, they are most commonly detected in acute myeloid leukemia and are linked to a poor prognosis along with decreased overall survival (Sun *et al*., 2018). Because DNMT3A mutations produce immortalized stem cells, they are sometimes associated with positive health outcomes in the elderly (Jeong *et al*., 2017). Research has suggested that these mutations might not solely lead to direct leukemic transformation, but rather form the premalignant state for hematological malignancy (Sun *et al*., 2018; Corces-Zimmerman *et al*., 2014).

Tet methylcytosine dioxygenase 2 (TET2) is a gene that catalyzes the conversion of methylcytosine to 5-hydroxymethylcytosine with the cofactor molecule α-ketoglutarate (α-KG) (Solary *et al*., 2014). The TET2 protein is involved in regulating the process of transcription and DNA demethylation (Feng *et al*., 2019). Although the encoded protein is ubiquitous throughout the human body, research suggests that it has a specific function in the formation of blood cells from hematopoietic stem cells (Solary *et al*., 2014). It appears that the protein acts as a tumor suppressor, thus regulating cells during division and replication. Defects in the gene are associated with several myeloproliferative disorders and acute myeloid leukemias (Feng *et al*., 2019). TET2 inactivation in mice has revealed hematopoietic defects. Similar to the DNMT3A mutation, TET2 protein misfolding has a unfavorable prognostic indication (Feng *et al*., 2019). Published data from Wang *et al*. (2019) in the journal *BMC Cancer* have indicated that AML patients will on average have a TET mutation varying from 6.05 to 27.36% and from 6.05 to 36.23% in patients with normal cytogenetics.

LEUKEMIA

1.61 Leukemia and Hematopoiesis

The study of leukemia has always been at the forefront of cancer research. It is a disease of early blood-forming cells and originates in the bone marrow. Leukemic cells may crowd out or suppress the development of normal cells leaving the individual prone to infection (Duarte *et al*., 2018). The rate of progression (acute vs chronic) and specific blood cell affected (myeloid vs lymphoid) are different with each type of leukemia

(Mackey & Klemm, 2000). Healthcare professionals have therefore categorized leukemia into four broad categories: acute lymphocytic, acute myeloid, chronic lymphocytic, and chronic myeloid (Mackey & Klemm, 2000). In total, the UPMC Hillman Cancer center website states that leukemia affects 45,000 people in the United States each year ("Leukemia," 2016). The NIH website estimates that as of 2016, over 400,000 individuals are living with some form of the disease ("Cancer Stat Facts: Leukemia," 2016). Because of the heterogeneity of the cancer, no two patients are alike, and the treatment and response can vary greatly (Li *et al*., 2016). Therefore, it is crucial for scientists to understand the pathogenesis of the disease and analyze each subtype of leukemia separately.

Hematopoiesis serves as a key model system to understand the basics of leukemia. The term simply means the process by which the body manufactures blood cells. All these cells develop from hematopoietic stem cells (HSCs) that can produce any other blood component (Gulati *et al*., 1988). Each of these components fall into one of three broad categories: erythrocytes, leukocytes, and thrombocytes. Red blood cells carry oxygen to tissues throughout the body, while white blood cells help fight infection and platelets form blood clots that control bleeding (Kuhn *et al*., 2017; King *et al*., 2018). The process of hematopoiesis initiates with an unspecialized stem cell in the bone marrow (Gulati *et al*., 1988). These stem cells transform into common lymphoid and myeloid progenitors to create two distinct pathways (see Figure 3). The common lymphoid progenitors further differentiate into separate lymphoblasts that help form the immune system. Lineage of the common myeloid progenitors separate into red blood cells,

platelets, or one of several types of white blood cells (Kondo 2010). Because the disease can originate in either lymphoid or myeloid lineage, the various forms of leukemia are grouped specifically on the type of white blood cell affected and the rate at which symptoms develop (Mackey & Klemm, 2000).

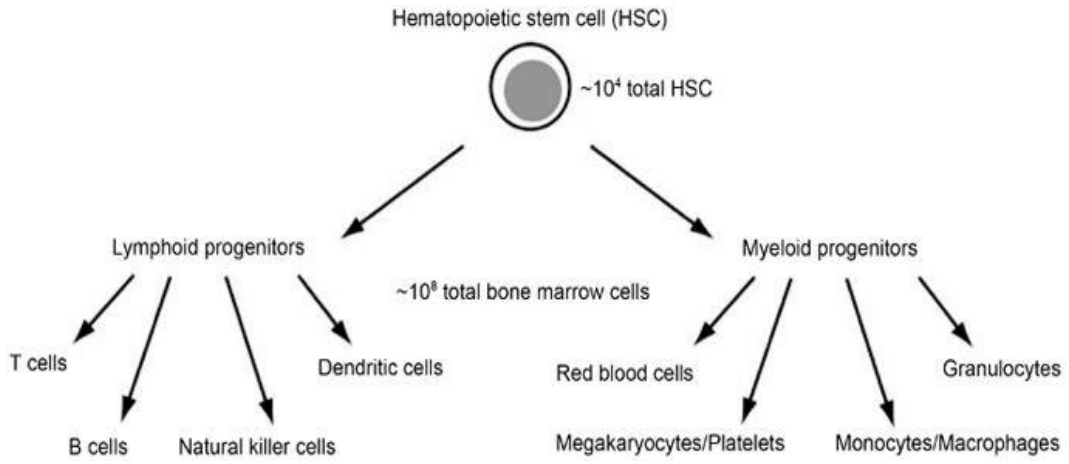

Figure 3: **Diagram of Hematopoietic Hierarchy**. Development of different blood and immune cells originating from a common hematopoietic stem cell (HSC). (Adapted from Nemeth & Bodine, 2007)

The majority of blood cells mature in the red spongy tissue of the bone marrow and then move into the peripheral vessels (Kuhn *et al*., 2017). In a leukemic individual, there is an abnormal amount of white blood cells that overtake the bone marrow. These overly abundant cells are not functioning as properly and are consuming more energy than their normal counterparts (Mackey & Klemm, 2000). Hence, the bone marrow is not able to produce enough of the normal blood cells. When the leukemic cells have no more space in the bone marrow, they eventually leak into the bloodstream (Kuhn *et al*., 2017). These events create a host of disorders such as anemia, thrombocytopenia, and leukopenia (Jalaeikhoo *et al*., 2017). Since leukemic cells are able to spread throughout

the body, the digestive tract, kidneys, lungs, heart are also affected (Luciano & Brewster, 2014; Ebert & Hagspiel, 2012). A common mechanism to diagnose leukemia is through a routine blood test and bone marrow biopsy (Béné *et al*., 2015).

1.62 Four Main Types of Leukemia

Leukemia is classified by the type of white blood cell affected and by how quickly the disease progresses (Mackey & Klemm, 2000). Uncontrolled proliferation of partially developed blast cells is a defining feature of acute leukemia while large amounts of morphologically mature, but functionally incompetent hematopoietic cells are recognized as chronic leukemias (Ladines-Castro *et al*., 2016). Acute leukemia transforms rapidly and becomes severe quickly whereas chronic leukemia grows more gradually and takes longer to progress (Albrecht 2014; Parikh 2018). Because the disease is either myeloid or lymphoid origin and either chronic or acute, the World Health Organization recognizes four main groups of leukemias (see Table 1). These characteristics of the disease are implemented to designate a patient with either acute myelogenous, acute lymphocytic, chronic myelogenous, or chronic lymphocytic leukemia.

Table 1**: Leukemia Categorization.** Four main types of leukemia and their particular attributes (adapted from Rafiq *et al*., 2018).

Type	Cells Involved	Cytology	Symptoms	Statistics
CLL	Lymphoid B or T cell	Chromosomal abnormalities	Swelling of lymph nodes and spleen	Common over 55 years of age
ALL	Immature B or T cells	Chromosomal aberration	Bone marrow dysfunction	Most common in children
CML	Pluripotent hematopoietic stem cell	Chromosomal translocation	Anemia, low platelet count	Rare in children
AML	Immature myeloid lineage cells	Epigenetic mutations	Anemia, excess bleeding	More common in adults

1.621 Chronic Lymphocytic Leukemia (CLL)

Chronic lymphocytic leukemia is a particular type of cancer for which the bone marrow produces too many mature lymphocytes. It is one of the most common types of leukemia in adults and often appears during or after middle age (Siegel *et al*., 2015). The lymphoproliferative disorder has morphologically mature, but functionally incompetent B-cells (Kipps *et al*., 2017). Clinically, CLL is asymptomatic, as the signs of the disease develop slowly in the course of several years (Hus & Roliński, 2015). Unlike other leukemias, the prognostic nature of CLL depends specifically on the stage of the cancer (Kipps *et al*., 2017). Because these cancer cells are mature, they travel with the normal lymphocytes to the lymph nodes, liver, and spleen (Schwartz & Shamsuddin, 1981). Over time these organ systems swell due to the accumulation of these cells (Hus & Roliński, 2015). There is a genetic component to the disease and risk factors include certain hazardous chemicals (Brown 2008). Immunophenotyping and flow cytometry are used to

confirm the diagnosis of CLL (Parikh 2018). There is unfortunately no cure for this leukemia, but because of the slow progression of the disease, it is not immediately life threatening (Dighiero & Hamblin, 2008; Parikh 2018).

1.622 Acute Lymphocytic Leukemia (ALL)

Acute lymphocytic leukemia is the result of mutations in precursor T and B blood cells usually due to a chromosomal translocation or abnormal chromosome number (Terwilliger & Abdul-Hay, 2017). These genetic circumstances generate atypical cellular proteins which regulate cell division. ALL is the most common leukemia overall and the most common cancer in children (Smith *et al*., 2010). Risk factors of the disease include antineoplastic agents, ionizing radiation, and Down syndrome (Belson *et al*., 2007). There is a bimodal distribution of those presenting with ALL as the median age of onset is 35 years with a large peak at 4-5 years of age and a smaller gradual increase in the elderly (Terwilliger & Abdul-Hay, 2017). The prognosis of the disease in children is favorable (>90%) while meager in adults (Smith *et al*., 2010). Because of the rapid nature of the disease, ALL – unlike CLL – is fatal if left untreated for even a few weeks or months. Fortunately, treatment of ALL is curable depending on the prognostic factors of the individual (Terwilliger & Abdul-Hay, 2017).

1.623 Chronic Myeloid Leukemia (CML)

Chronic myelogenous leukemia is a cancer of all of the differentiated white blood cells except the lymphocytes (Granatowicz *et al*., 2015). Because of the nature of a chronic leukemia, it is a monoclonal disorder of late – small, mature, but dysfunctional – hematopoietic stem cells. The onset of CML is insidious and patients are typically older,

with a median age of 40-50 years (Huang *et al*., 2012). Karyotype analysis of the disease presents with a balanced reciprocal translocation of the proto-oncogene ABL with a break cluster region resulting in a characteristic change known as the Philadelphia chromosome (Serra *et al*., 2014). It results in the bone marrow assembling protein, called tyrosine kinase, that triggers too many hematopoietic stem cells to become white blood cells (Granatowicz *et al*., 2015). Therefore, current treatment of the disease is with a tyrosine kinase inhibitor which has led to drastically improved survival rates (Huang *et al*., 2012; Pophali & Patnaik, 2016). Individuals with an early phase of the disease can expect a normal life expectancy with Imatinib treatment (Kim *et al*. 2011).

1.624 Acute Myeloid Leukemia (AML)

Acute myeloid leukemia is a cancer of the myeloid lineage characterized by uncontrolled, rapid growth of partially developed white blood cells that build up in the bone marrow (Lin & Smith, 2011). Risk factors of AML include antineoplastic agents, ionizing radiation, and benzene exposure (Tsai *et al*., 2014). AML has several different subtypes for which prognosis and treatments may vary. Diagnosis of the disease is based on morphologic investigation, cytogenetic studies, molecular markers, and cytochemical analysis (Lin & Smith, 2011). Death occurs within weeks to months unless a first remission is achieved (Foster *et al*., 2015).

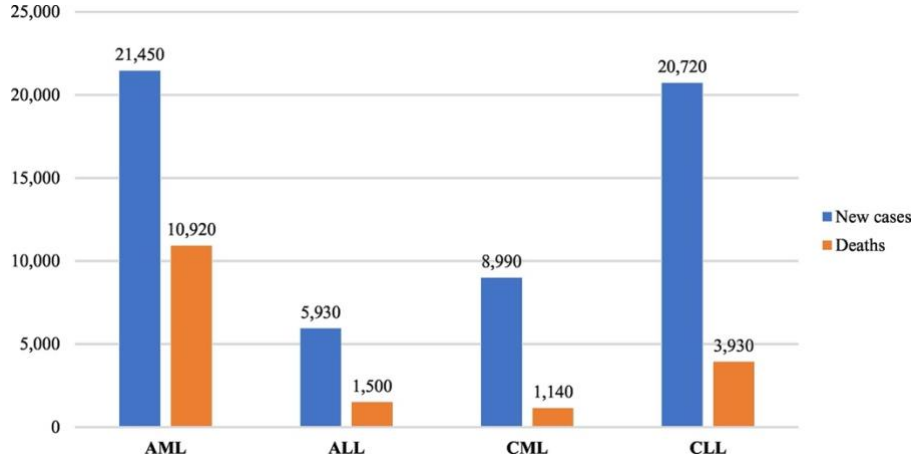

Figure 4: **Statistics on Leukemias**. The 2019 estimated cases and deaths from the four largest subtypes of leukemias. Acute myeloid leukemia has the worst prognosis with the most fatalities. (Adapted from Shallis *et al*., 2019)

ACUTE MYELOID LEUKEMIA

1.7 Prognostic Challenges

Modern knowledge of acute myeloid leukemia has only amplified the sense of medical helplessness of the disease because until 2017 previous treatment has not improved prognosis. This cancer has been studied in exquisite detail, but without any therapeutic or practical advances until recently (Watts & Nimer, 2018). Whole genome sequencing has allowed researchers to detect mutation frequency in cancer. However, the relationship between potential driver mutations and epigenetic phenotypes in AML patients are not yet clear (Li *et al*., 2016). Furthermore, defining nearly all coding mutations in AML at initial presentation has not solved risk assessment of the disease (Moarii & Papaemmanuil, 2017). The evaluation of this cancer must involve several confounding variables as subclones of any individual with AML have unique functional and morphologic properties (Spencer *et al*. 2014).

Although AML is a relatively rare cancer in the general population, it accounts for 90% of leukemias in all adults and has the poorest modeling of all of the leukemias (Jemal *et al*., 2002). The cytogenetic and molecular characterization of the disease are complex and not well defined (Spencer *et al*., 2014). There are several dimensions of AML which may explain its imperfect risk assessment at initial presentation. The mutational and clonal intricacy along with its variable evolution over time have given the cancer many paths to resistance from standard chemotherapy (Martignoles *et al*., 2018). More recent investigations of AML have suggested that epigenetic signatures of the cancer might play the biggest role in the disease pathogenesis of many AML patients (O'Brien *et al*., 2014).

The prognosis of AML is starkly different than other forms of leukemia. The fast nature and target population of AML reflects its clinical importance (Ley *et al*., 2010). Chronic leukemias may require no treatment due to the late and slow onset of the disease (Hus & Roliński, 2015). Although untreated acute leukemias are devastating, there are positive therapeutics to slow down the disease if it is of a lymphoid origin and the individual is young of age (Terwilliger & Abdul-Hay, 2017). However, AML is an age associated cancer with an overall 5 year survival rate of less than 5% in patients over 65 years of age (Almeida & Ramos, 2016). Because of these statistics, coupled with the slow therapeutic advances and challenges, there has recently been a resurgence in the awareness and investigative passion of the disease within the healthcare community.

1.72 Epidemiology of Acute Myeloid Leukemia

1.721 Incidence

The Surveillance, Epidemiology, and End Results (SEER) program of the National Cancer Institute (NCI) gathers and publishes information on cancer statistics. It is one of the largest cancer registrars covering approximately 34.6% of the United States population. The initial SEER reported that age-adjusted AML incidence within the U.S. alone ranges from three to five cases per 100,000 population. In addition, the disease is the most common acute leukemia in adults despite representing 1.2% of all new cancer cases (Maksimovic *et al*., 2018). The lifetime risk of developing AML is approximately 0.5% according to the SEER 9 database. Moreover, an estimated 61,048 people are living with acute myeloid leukemia in the United States (Data from SEER 9). Notably, the incidence rate per 100,000 has been slightly rising in the United States due to a global aging population (see Figure 5) (Shallis *et al*., 2019). The age-adjusted prevalence rates in the United Kingdom, Canada, Australia, Sweden, as well as Denmark parallel those of the US population suggesting that a model for the pathogenesis of the disease is based on a group of fixed variables (Shallis *et al*., 2019).

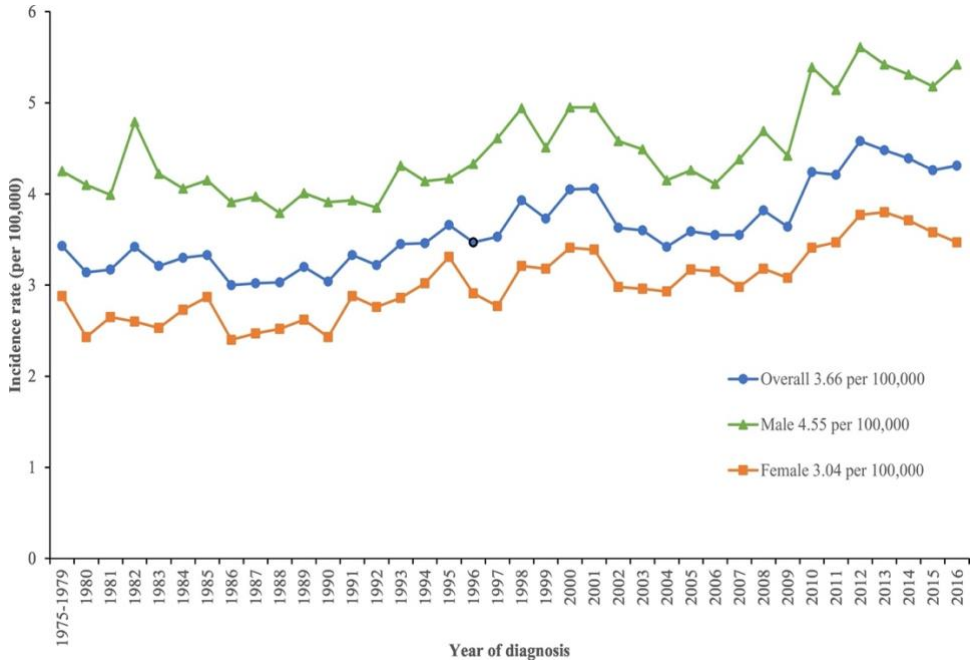

Figure 5: **The Incidence of AML**. The occurrence of the disease in the United States has been slightly rising since 1975 but not every year. The overall general increase of AML patients is probably due to an aging US population. (Adapted from Shallis *et al.*, 2019)

1.722 Age

Acute myeloid leukemia is primarily a disease of age (Almeida & Ramos, 2016). While the disease is diagnosed at any age, it is uncommon in those under 45 years of age. The incidence of those aged 65 years or older is 20.1 per 100,000 while 2.0 per 100,000 for those that are younger (SEER Cancer Statistics Review 1975-2016). The median age of initial diagnoses is 68 years of age in the United States (see Figure 6) (SEER Database 2001-2013). Because of the late onset of the cancer, many patients are unable to obtain the standard intensive therapies due to other co-existing medical conditions (Maher *et al.*, 2013). Worse, the disease itself tends to act more aggressively the further one ages due to separate comorbidities (López-Otín *et al.*, 2013). These factors have confronted healthcare professionals with the age-related challenges of AML.

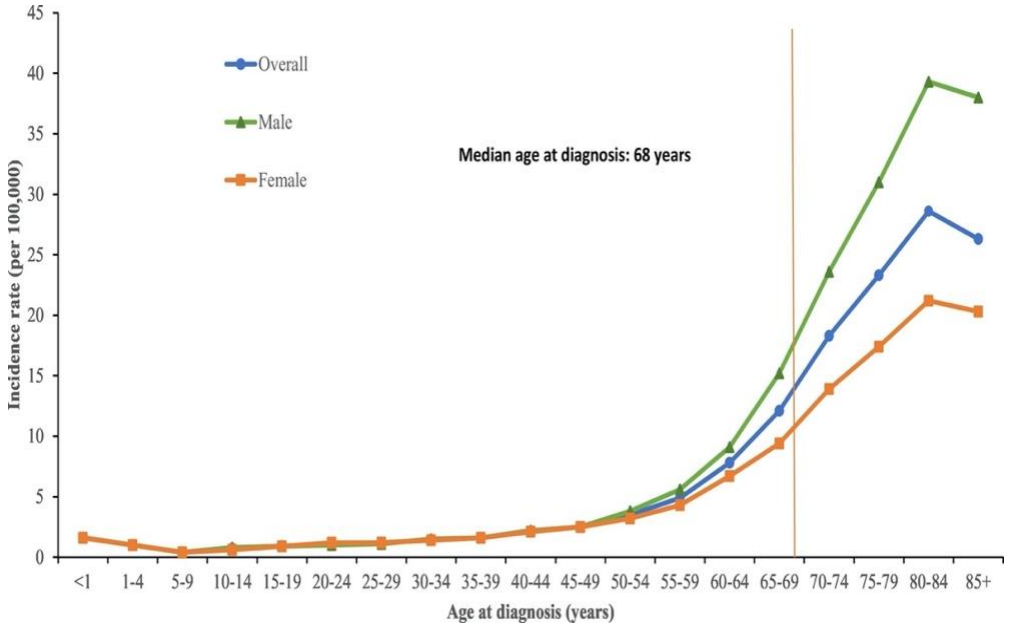

Figure 6: **Incidence of AML Increases with Advanced Age**. The median age in the United States is 68 at diagnosis (Adapted from Shallis *et al*., 2019).

1.723 Gender and Race/Ethnicity

Acute myeloid leukemia occurs in all racial groups (Kannan *et al*., 2014). Males across all ethnicities are 1.2-1.6 times more likely to develop AML during their lifetime according to the SEER data. Studies in the US, UK, and Canada have an age adjusted incidence of 5.42 and 3.47 per 100,000 individuals in males and females respectively (Shallis *et al*., 2019). Native Americans have the highest incidence rate while Caucasians have the lowest (Shallis *et al*., 2019). The exact mechanism accounting for these disparities is unclear but suggests a hormonal and social factor might play a role in the acquisition of the disease and its pathogenesis (Poynter *et al*., 2013). Because of the challenges in establishing the true prevalence of the disease, many studies consider race a confounding variable in AML (Kannan *et al*., 2014).

1.73 Diagnosis and Risk Classification

The diagnosis of AML is often tricky and not standardized as individuals present initially with unspecific symptoms such as fatigue, chronic inflammation, and muscle weakness (Albrecht 2014). Evaluation and initial workup of someone suspected of acute myeloid leukemia involves a comprehensive medical history with a physical checkup followed by a routine blood sample (Béné *et al*., 2015). The initial diagnosis of leukemia is often quick via a blood smear, but further classification of the cancer requires a bone marrow aspiration and biopsy with cytogenetic analysis (Béné *et al*., 2015). These tests are employed to identify specific genes, proteins, and other factors such as translocations involved in the cancer. DNA sequencing and cytogenetics are used to classify the AML subtype which will guide prognosis and treatment (Grimwade *et al*., 2010).

1.74 Morphologic Classification

Morphology of AML is determined by the identification of the leukemic cell line and its stage in differentiation (Ladines-Castro *et al*., 2016). The mechanism to assess this is through the peripheral blood, bone marrow, and less commonly in solid tissues (Luciano & Brewster, 2014; Ebert & Hagspiel, 2012). Myeloid blast cells appear small to medium in size with high nucleo:cytoplasm (n:c) ratio and dispersed chromatin (Ladines-Castro *et al*., 2016). Distinct from other acute leukemias, Auer rods (see Figure 7) present themselves in AML, which is a defining hallmark of the cancer (Willis *et al*., 2005; Bain 2011). In addition, the histological presentation of the disease further includes increased bone marrow cellularity, consisting primarily of granulocytic or monocytic forms and variable numbers of erythroid precursors (Orazi 2007). Immunophenotyping can also

supplement the diagnosis of specific genetic categories of acute myeloid leukemia (Klco *et al*., 2014).

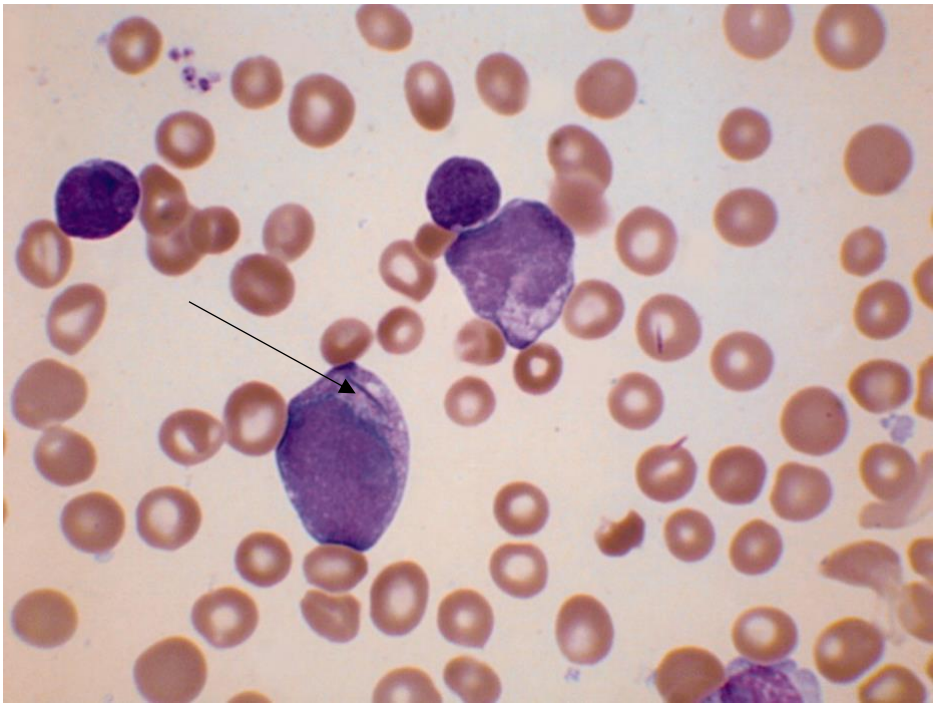

Figure 7: **White Blood Cell Differential**. The medical laboratory test provides information about the types and amounts of white blood cells. Someone with acute myeloid leukemia will typically have a blood smear containing an Auer body (arrow) – a hallmark of the disease. An Auer body is an azurophilic needle shaped granule observed in myeloid blast cells. (Adapted from Bain 2011)

1.75 Cytogenetic Abnormalities and Pathogenesis

Cytogenetic analysis of AML has been studied to analyze the molecular pathogenesis of the disease. Recurrent chromosomal structural variations in AML individuals have established diagnostic and prognostic markers (Welch *et al*., 2012). However, defining all of the mutations of the disease has not improved its risk assessment (Ley *et al*., 2010). Moreover, approximately 50% of AML patients have a normal karyotype that lack structural abnormalities and no driver mutations in any of the

recognized driver genes of the disease (Akagi *et al.*, 2009). Interestingly AML genomes have the fewest mutations of all adult cancer types with only 23 significantly mutated genes (see Figure 8) when corrected for genome size (Marchesi 2013). The most affected recurrent genes include FLT3, NPM1, and DNMT3A because they tend to cooccur (Wang *et al*., 2017). Because of the complexity of the disease, there are several confounding variables and dimensions of AML (Ley *et al*., 2010).

The AML genomes to date are clonally complex with no exception. They all consist of a founding clone with different multigenerational daughter subclones (Martignoles *et al.*, 2018). Hematopoietic stem cells divide once a month and develop one mutation for each division (Kovtonyuk *et al*., 2019). Therefore, as an individual ages, each progenitor stem cell has a genetic signature with a unique characteristic pattern of gene expression. Preleukemic mutations develop over time and slightly skew clonal mutations to a founding clone that favor cancer such as abnormalities in FLT3, NPM1, and DNMT3A genes (see Figure 8) (Martignoles *et al*., 2018). The biggest problem with the founding clone is that it experiments with additional, upstream mutations to produce more subclones for which there are many in an individual with AML (Klco *et al*., 2014). These mechanisms have the potential to explain the explosive, accelerative nature of the disease.

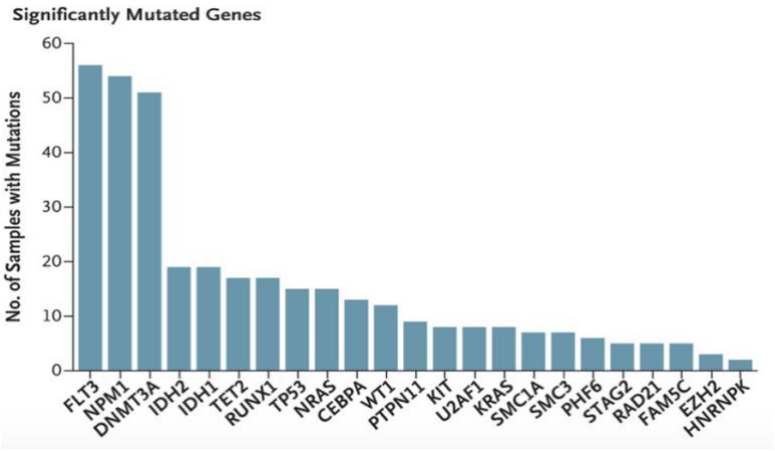

Figure 8: **The Big Three Genes: FLT3, NPM1 & DNMT3A**. There are 23 notable mutated genes in acute myeloid leukemia when corrected for gene size. These genes tend to cooccur in individuals with AML. In the total distribution, there were 260 recurrently mutated genes and 1627 singletons found in the cohort. (Adapted from Ley *et al*., 2013)

There has been only one robustly correlated gene linked to AML and it is the DNMT3A R882 gene (which encodes DNA methyltransferase A) (Ketkar *et al*., 2020). The presence of this defected gene independent of FLT3 or NPM1 mutations has been associated with a worse outcome in those individuals with AML as it has an important role in chemoresistance (Guryanova *et al*., 2016). It is believed that the abnormal DNMT3A R882 gene is the first in a series of mutations representing the founding clone of the cancer. However, the relationship between DNMT3A and AML is unclear as these mutations do not cause genomic instability nor change 5-methylcytosine content (Ley *et al*., 2010). Tien et al. (2014) in a sample of 500 AML patients identified DNMT3A mutations in 14% of them while 22.9% had a normal karyotype. The presence of this very specific mutation may provide a new tool to assess the pathogenesis of AML, but is not a causal factor in the development of the cancer (Ley *et al*., 2010). However, a

precise characterization of clonal architecture in AML has important clinical implications.

1.76 Initiating Mutation and Clonal Skewing

The initiating mutations of AML have been extensively covered in the literature as the disease is clonally complex (Martignoles *et al.*, 2018). There is on average one mutation per cell division of hematopoietic stem cells, which engender different types of blood cells in lines called myeloid or lymphoid (Kovtonyuk *et al.*, 2019). Because these hematopoietic stem cells are long-lived for several decades and divide once a month, each hemopoietic stem cell over time has an accumulation of mutations along with their own unique genetic signature (O'Brien *et al.*, 2014). Each of these mutations separately are considered insignificant, but some produce a preleukemic advantage (Ley *et al.*, 2010). Certain hematopoietic stem cells are able to then divide quicker. Research has shown that these mutations are typically in the FLT3, NPM1, TET2, and DNMT3A genes. These initiating mutations in AML tend to skew clonal hemopoiesis in elderly patients to a single clone (Ley *et al.*, 2010). Expressed in a different way, individuals with AML or clonal hematopoiesis tend to have the same mutations, thus anchoring these concepts together (see Figure 9) (Xie *et al.*, 2014). However, the data only represents a correlation and not a causation.

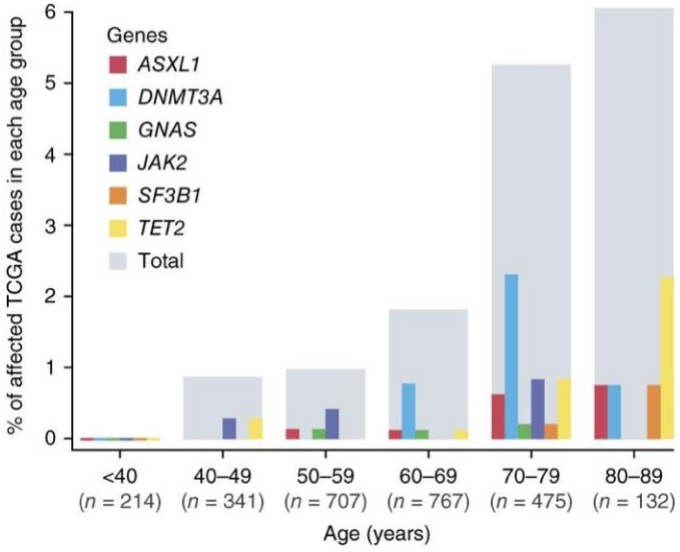

Figure 9: **Clonal Skewing with AML Initiating Mutations**. The amount of mutations in 6 cancer associated genes increase exponentially with age. These are recurrent mutations that are tend to cooccur and coexist. However, cooperation of these genes remains unclear. (Adapted from Xie *et al*., 2014)

The initiation of acute myeloid leukemia probably begins with a DNMT3A mutation followed by a NPM1 and FLT3 mutation (Ley *et al*., 2010). Subclones then exponentially expand after the acquisition of all of the mutations, thus defining the explosive nature of the disease. These leukemic cells have been desensitized to the massive proliferative signal and are clustered together (Ley *et al*., 2010). Subclones of AML are extremely important due to the hierarchy of the disease (Bonnet & Dick, 1997). Moreover, subclones have distinctive functional and morphologic properties such as altered growth properties *in vitro* as well as unique immunophenotypes (Klco *et al*., 2014). NPM1 and FLT3 inhibitors are incapable of effectively treating those with AML because the founding close still is unaffected and able to more produce more subclones (Ley *et al*., 2010). Therefore, relapse is likely to occur. Hence, current research suggests

targeting the founding clone, which may begin with the DNMT3A mutation (see Figure 10) (Walter *et al.*, 2013; Welch *et al.*, 2012).

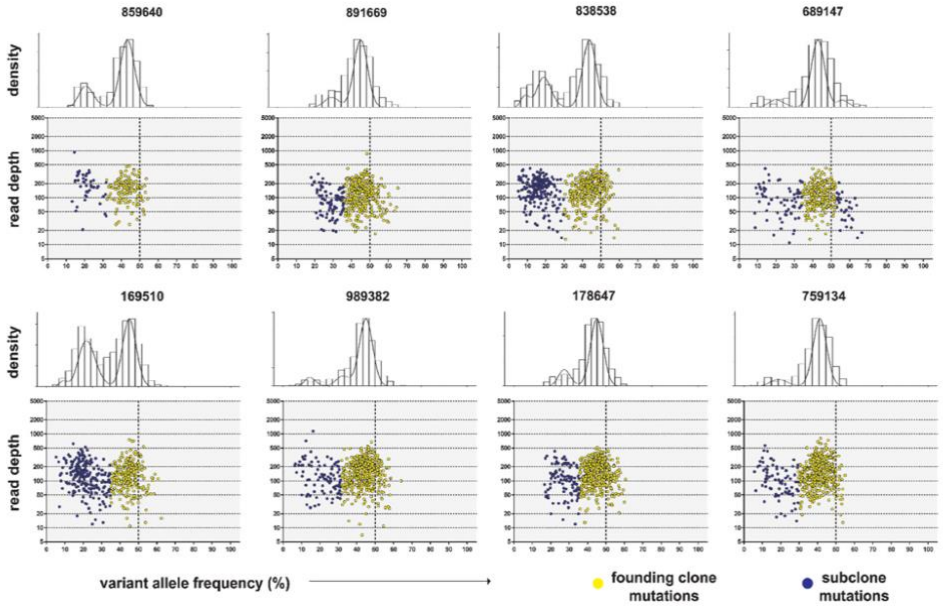

Figure 10: **Clonal Diversity of AML**. Variant Allele Frequency (VAF) vs read depth of 8 AML genomes. Founding clones produce variable subclones. The histogram above each scatter plot show clustered VAFs using a Bayesian approach. Higher amplitude peaks on the histograms represent an initial DNMT3A mutation in the founding clone while the smaller amplitude peaks represent additional, but later mutations in either IDH1, FLT3, or both for the subclones. (Adapted from Walter *et al.*, 2013).

1.77 Treatment

The main treatment for AML is cytotoxic chemotherapy, but it is sometimes combined with targeted drug therapy (Lai *et al.*, 2019). Standard of care is branched into two phases: induction of remission and consolidation therapy. The objective of induction therapy is to decrease the tumor burden to an undetectable level with traditional chemotherapeutic agents (Lai *et al.*, 2019). The standard chemotherapy medication to treat AML is cytarabine, which is a pyrimidine nucleoside analog that inhibits the synthesis of DNA (Lee *et al.*, 2000). Cytarabine is given intravenously in a single dose

for seven straight days then followed by daunorubicin or idarubicin (anthracycline drugs) for another three days. This is sometimes called the "7+3" regimen (cytarabine 7 days + daunorubicin or idarubicin 3 days) (Lichtman 2013). These drugs are highly effective and frequently result in complete remission of leukemia – meaning no visible AML cells are seen in the blood or bone marrow when examined under a microscope (Lai *et al*., 2019). However, these tumor-free remissions are temporary because leukemic cells begin to regrow afterwards and become better equipped to evade these chemotherapeutic medications (Lee *et al*., 2000).

Post remission therapy immediately follows induction treatment. The standard of care is usually another dosage of cytarabine or either a stem cell transplant depending on the current risk assessment (Lai *et al*., 2019). Nonetheless, relapse is likely to occur within one to two years after starting the initial treatment (Lee *et al*., 2000). There are currently several experimental immunotherapies to target AML for post remission therapy. The main treatment is via allogenic stem cell transplantation from a healthy donor (Peccatori & Ciceri, 2010). However, the risk of side-effects are life-threatening due to the complication of graft vs host disease. Fortunately, there has been an explosion of new FDA-approved medications (see Figure 11) that can potentially target acute myeloid leukemia (Winer & Stone, 2019).

The past several decades have seen little improvement in the treatment of AML (Lai *et al*., 2019; Cai & Levine, 2019). Chemotherapeutic agents such as cytarabine and daunorubicin are still the standard of care (Lee *et al*., 2010). These medications were first described in 1975 and have been used continuously since 1981. A complete remission

rate with the "7+3" treatment is 60-85% for those younger than 60 and a meager 40-60% for those older than 60 years of age with AML (Rai *et al*., 1981). The reason for the lack of advancement and outcome with respect to AML treatment for over 40 years stems from an incomplete screening mechanism for new leukemic genes (Ley *et al*., 2010). Moreover, healthcare professionals have not successfully targeted the initial mutation(s) that potentially engender acute myeloid leukemia. Fortunately, the emerging field of epigenetics may hint at a new direction to better understand the complexities of AML, resulting in better therapeutic invention (Goldman *et al*., 2019).

Drug	Date of approval	Indication
Midostaurin (Rydapt) Novartis	28 April 2017	Treatment of adult patients with newly diagnosed AML who are FLT3+* in combination with standard cytarabine and daunorubicin induction and cytarabine consolidation
Enasidenib (Idhifa) Celgene	1 August 2017	Treatment of adult patients with relapsed or refractory AML with an isocitrate dehydrogenase-2 (IDH2) mutation*
CPX-351 (Vyxeos) Jazz Pharmaceuticals	3 August 2017	Treatment of adults with newly diagnosed therapy-related AML (t-AML) or AML with myelodysplasia related changes (AML-MRC)
Gemtuzumab ozogamicin (Mylotarg) Pfizer	1 September 2017	Treatment of adults with newly diagnosed CD33-ositive AML and for treatment of relapsed or refractory CD33-positive AML in adults and in pediatric patients 2 years and older. May be used in combination with daunorubicin and cytarabine for adults with newly diagnosed AML.
Ivosidenib (Tibsovo) Agios	20 July 2018	Adult patients with relapsed or refractory AL with a susceptible IDH1 mutation*
Glasdegib (Daurismo) Pfizer	21 November 2018	In combination with low-dose cytarabine for the treatment of newly diagnosed AML in adults who are aged 75 years or older, or who have comorbidities that preclude use of intensive induction chemotherapy.
Venetoclax (Venclexta) Abbvie/Genetech	21 November 2018	In combination with azacitidine or decitabine or low-dose cytarabine for the treatment of newly diagnosed AML in adults who are aged 75 years or older, or who have comorbidities that preclude use of intensive induction chemotherapy.
Gilteritinib (Xospata) Astellas Pharma	28 November 2018	Treatment of adult patients who have relapsed or refractory AML with a FLT3 mutation*

Figure 11. **Recent FDA Approvals for AML**. There is great activity in the field of AML as 8 new drugs have been approved to target new or relapse subtypes of the disease. (Adapted from Leukemia & Lymphoma Society of Canada: Acute Myeloid Leukemia – Diagnosis and Treatment in 2020)

CHAPTER 2

OBJECTIVES

The purpose of the thesis is to better characterize the DNMT3A gene as an epigenetic driver in clonal hematopoiesis and acute myeloid leukemia. In order to address the objective, WT and mutant DNMT3A proteins will be generated via a bacterial expression vector.

CHAPTER 3

METHODS

3.1 Overview

The goal of the research was to generate DNMT3A proteins for future research via a bacterial expression vector. A wild type (WT) and mutated version (W306C) of the DNMT3A cDNA were amplified via multiple polymerase chain reactions. In order to produce the proteins of interest, versions of the DNMT3A cDNA were ligated into a pGEX expression vector. E. coli bacteria were then transformed with the expression vector and with the cell's machinery produced the DNMT3A protein encoded by the DNMT3A cDNA. The expression vector encodes an antibiotic resistance cassette to choose the colonies that have incorporated the DNMT3A cDNA. The strategy of generating the DNMT3A gene's targeted protein via an expression vector is outlined below.

3.2. Obtain the DNMT3A Sequence

Polymerase Chain Reaction (PCR) was the method of amplifying cDNA. The process is used very quickly and efficiently produces millions of copies of a single segment of cDNA. The ingredients of a successful PCR reaction include (1) the target DNA molecule (DNMT3A), (2) the pair of DNA primers, (3) heat-resistant DNA polymerase, and (4) deoxyribonucleotide triphosphates (dNTP's). There was a total of four reactions – two templates (WT and W306C DNMT3A) and two primer pairs (F1/R and F2/R). The double strands of DNA were heated at 95° C, breaking the electrostatic

hydrogen bonds holding them together. Then the PCR solution was cooled to 55° C, allowing hybridization to reoccur. The temperature was afterwards increased to an optimal 72° C, where the DNA polymerase was able to add the dNTPs to elongate the strand in the 5' to 3' direction.

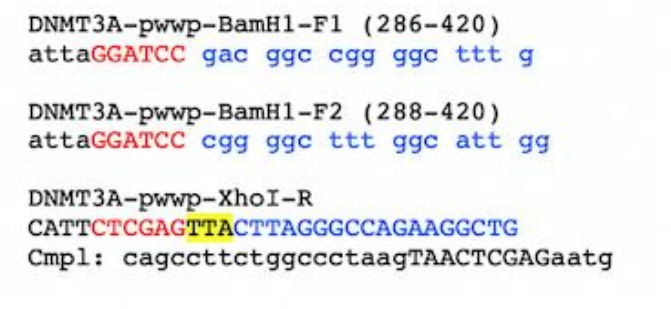

Figure 12: **PCR Primer Design and Amplification of DNMT3A PWWP Domain**. Two forward primers were designed as the GC content at 5' of PWWP domain is high, which is potentially difficult to amplify. F1/R amplifies DNMT3A residues 286-420 while F2/R amplifies DNMT3A residues 288-420. BamH1-F and XhoI-R primers were chosen in order to replicate a specific cDNA sequence of the DNMT3A mRNA that can be incorporated into a bacterial expression vector after BamH1 and XhoI digestion.

3.3. Ligating cDNA into the pGEX vector

The PCR products were incorporated into a bacterial vector via restriction digestion (BamHI and XhoI). These type II restriction endonucleases cut the pGEX plasmid at specific locations, facilitating the introduction of the cDNA fragments into the vector via a T4 DNA ligase.

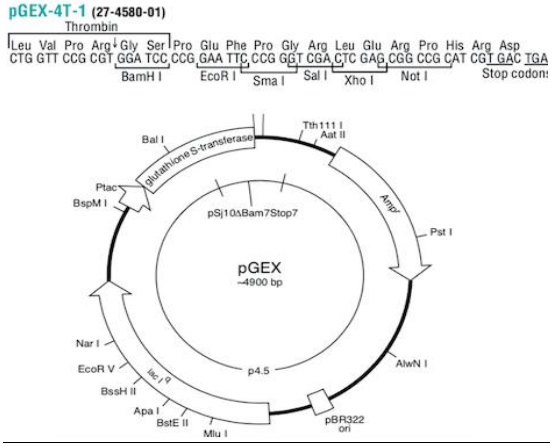

Figure 13: **Cloning GST-PWWP of DNMT3A**. In-frame cloning of the cDNA within the BamH1 and Xhol site. The vector consists of an insert and a larger sequence known as the backbone.

3.4 Screening

The DNMT3A cDNA (WT and 306A) were ligated to the pGEX vector and transformed into E. coli bacteria. These recombinant DNA vectors were able to replicate in an incubator. There were LB agar plates with AMP for either the DNMT3A WT pGEX vector, DNMT3A 306A pGEX vector, or pGEX backbone. These transformed bacteria were plated on LB+AMP plates rather than LB plates. The antibiotic used was designed to select the E. coli of interest that contained the plasmids.

CHAPTER 4

RESULTS

The major goal of the experiment was to generate DNMT3A proteins for future research via a bacterial expression vector. There is extensive literature to suggest that DNMT3A protein misfolding is associated with hematological malignancies specifically with acute myeloid leukemia in the elderly (Ley *et al*., 2010). Past research on the DNMT3A gene has not changed the prognosis of those suffering from leukemia because the clonal nature of the disease is complex (Spencer *et al*. 2014). A wild type (WT) and mutant cDNA version of DNMT3A were amplified via PCR. The fragment sizes of the cDNA fragments were measured via agarose gel electrophoresis (see Figures 14 & 15). The cDNA fragments (WT and W306C DNMT3A cDNA) were not successfully integrated into the pGEX bacterial expression vector. Moreover, purified DNMT3A proteins were not synthesized, but this is still an ongoing process. Millions of colonies were observed on the backbone plate (see Figure 17) thereby confirming a mistake in the conduction of the experiment.

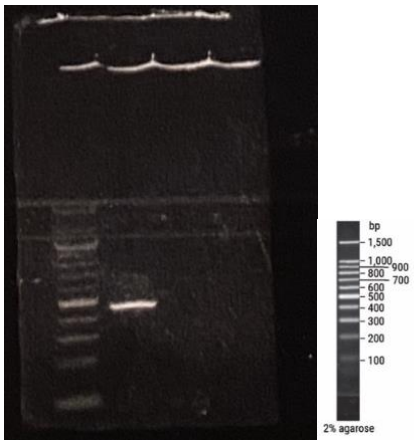

Figure 14: **WT DNMT3A cDNA Agarose Gel Electrophoresis**. Following PCR of WT DNMT3A cDNA, the fragments were separated based on size. The first column had Invitrogen 100 bp DNA Ladder which was used to size the number of base pairs of WT DNMT3A cDNA. Approximately 13 individual chromatography-purified DNA fragments were represented in the 100 bp DNA Ladder. The fragment length of WT DNMT3A cDNA with primers DNMT3A-pwwp-BamH1-F2 and DNMT3A-pwwp-XhoI-R was 400 base pairs (bp). A loading dye was placed in lane two to visually track DNA migration during electrophoresis.

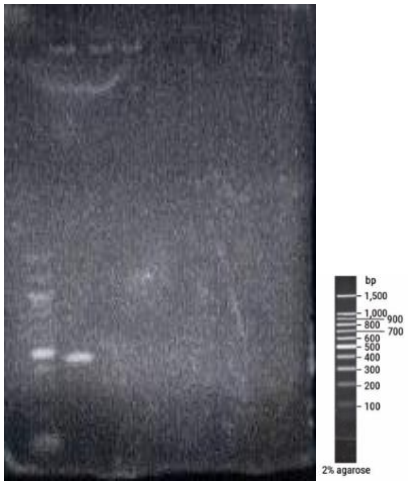

Figure 15: **W306C DNMT3A cDNA Agarose Gel Electrophoresis**. W306C DNMT3A cDNA fragments were separated based on size and compared to the Invitrogen 100 bp DNA Ladder reference. The fragment length of W306C DNMT3A cDNA with primers DNMT3A-pwwp-BamHI-F2 and DNMT3A-pwwp-XhoI-R was 420 base pairs (bp). A loading dye was placed in lane two to visually track DNA migration during electrophoresis.

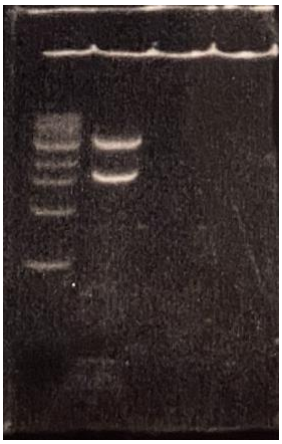

Figure 16: **pGEX-4T-1 Plasmid Agarose Gel Electrophoresis**. Type II restriction endonucleases (BamHI and Xhol) were supposed to cut the bacterial expression vector into separate two pieces. However, two visible bands on the agarose gel represented an error in the conduction of the experiment. Although the vector was cut, the fragment of DNA between the BamHI and XhoI was not visible as the segment contained few bases. Therefore the agarose gel should have had one fragment consisting solely of the backbone of the vector. Two bands here represented a mixture of linear DNA (upper band) and the uncut supercoiled DNA. Hence, the reaction was incomplete.

Figure 17: **E. coli Transformation and Selection**. There were millions of colonies on the three separate plates. The left petri dish contained the pGEX-4T-1 vector with the W306C DNMT3A cDNA while the right petri dish had the pGEX-4T-1 vector with the WT DNMT3A cDNA. The middle petri dish was used as the control and was not supposed to contain any insert. Unfortunately this was not the case as millions of colonies were present even on a non-ampicillin plate.

CHAPTER 4

DISCUSSION

Cancer treatment is one of the fastest growing specialties in modern medicine (Krzyszczyk *et al*., 2018). In-depth studies on cancer have advanced the understanding of the disease and have improved the ever-evolving therapeutic options. Nonetheless, scientists and researchers still encounter a wide translational gap between this new unprecedented knowledge and patient care because of the complex, dynamic nature of cancer (Ghoshal 2017). Great genetic diversity is seen in cancer as it is produced by competition among clonal lineages generated by somatic mutations (Ley *et al*., 2010). It is widely assumed that the greater the genetic diversity in a tumor, the more likely it will progress to malignancy. However, acute myeloid leukemia is a notable exception as it possesses relatively few mutations across the genome (Ley *et al*., 2010). The relative paucity of genetic aberrancy in acute myeloid leukemia might be a key critical reason in its cytotoxicity and poor prognosis. Therefore, the cure for a particular cancer cannot be based solely on its genetics.

The main challenge in cancer treatment is its clonal evolving nature. Nearly every known cancer originates from one ancestral stem cell that has the developed the capacity to renew indefinitely (Veith *et al*., 1987; Feinberg *et al*., 1985). Hence, there are limitless number of descendants with potential unbridled growth. Worse, the generations of each cancer lineage are different (Izaguirre *et al*., 1979). The genetic instability of cancer, coupled with its mirthless expansion, provide an almost an omnipotent weapon against the immune system and chemotherapeutic drugs. Cancer, as stated by Siddhartha

Mukherjee in his Pulitzer Prize winning book *The Emperor of All Maladies: A Biography of Cancer*, is the "ultimate product of Darwinian selection" (p. 51). The fitness of the disease evolves and increases after each subsequent generation (Smetana *et al*., 2019). Therefore, the cure of cancer begins with its prevention.

The prognosis of AML has been constant for several decades due to the complexity of the disease and failure to clear all variants associated with increased risk of relapse. Moreover, initiating mutations such as DNMT3A are more persistent and less likely to be cleared than cooperating mutations. The reason might stem from epigenomic alterations, specifically the DNMT3A R882 mutation that reduces methyltransferase activity in AML cells. Moreover, R882H AMLs have altered chromatin structure and very few canonical changes in gene expression (Klco *et al*., 2014). Knowledge regarding this specific mutation is in its early stages as it is difficult to detect despite deep DNA analysis. Because initiating mutations play an important role in cancer development, DNMT3A R882H is a major therapeutic target. New research has suggested that altering the WT:R882H heterodimer with a small molecule could potentially restore methylation activity and therefore may cure hematological malignancies as well as clonal hematopoiesis (Klco *et al*., 2014).

The purpose of this research project was to generate DNMT3A proteins for further research as it is an important regulator in clonal hematopoiesis and acute myeloid leukemia. Data gathered in the laboratory was incomplete. The primers used to amplify the cDNA were successful; however, the bacterial vector was incompletely cut. Hence, the control for the experiment - the petri dish that contained only the backbone DNA -

produced millions of colonies even on a non-ampicillin plate. Although the experiment is an ongoing process, certain steps must be performed again in order to generate the DNMT3A proteins of interest with the goal of characterizing them in the future.

There is a convergence of topics when discussing acute myeloid leukemia. The age-related component of the disease, along with the concept of clonal hematopoiesis, have suggested an error in the DNMT3A gene producing a mutant DNMT3A protein. Because the elderly population in the United States according to the Census Bureau website is estimated to more than double in the next several decades, the impact of a mutant DNMT3A gene will continue to rise. Therefore it is critical to restore function in this gene for those battling acute myeloid leukemia. In addition, further characterization of the DNMT3A gene will have an impact in cancer research, but more importantly critical care. A mutation in the DNMT3A gene is usually present in the founding clone of AML and therefore proper treatment should focus on this gene mutation rather than separate mutations in the subclones (Ley *et al*., 2013). If healthcare professionals successfully target and treat the DNMT3A mutation, the prognosis of acute myeloid leukemia might potentially improve.

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